Examination of the *FLT3* and *NPM1* mutational status in patients with acute myeloid leukemia from southeastern Poland

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Submitted: 8 January 2014
Accepted: 5 April 2014

Arch Med Sci 2016; 12, 1: 120–128
DOI: 10.5114/aoms.2015.49811
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Abstract

Introduction: Acute myeloid leukemia (AML) is a genetically heterogeneous disease at both the cytogenetic and molecular levels. In AML cells many chromosomal aberrations are observed, some of them being characteristic of a particular subtype of patients, and others being less significant. Besides chromosomal abnormalities, the leukemic cells can have a variety of mutations involving individual genes. The aim of this work was to investigate the frequencies of molecular alterations with the focus on *FLT3*-ITD and *NPM1* mutations in AML patients of different age groups living in a southeastern region of Poland.
Material and methods: The study group comprised 50 consecutive AML patients. We analyzed bone marrow samples by conventional cytogenetics. Cytogenetic evaluation in selected cases was complemented by the FISH technique. The internal tandem mutation in the *FLT3* gene was identified using polymerase chain reaction (PCR), and the *NPM1* mutation was assessed by direct nucleotide sequencing.
Results: The studies using classical cytogenetics showed chromosomal aberrations in 32 (64%) patients. In 18 cases no changes in the karyotype were found by conventional karyotyping. *FLT3*-ITD mutation was detected in 4 (8%) patients and mutation of *NPM1* in 3 patients with AML (6%).
Conclusions: The incidence of both mutations in our study group was lower than described elsewhere. We have confirmed that *FLT3*-ITD occurred more commonly in older patients and it was associated with shorter overall survival. By contrast, mutation of exon 12 of the *NPM1* gene seems to be a good prognostic factor in AML patients with normal karyotype.

Key words: acute myeloid leukemia, *FLT3*-ITD, *NPM1*, karyotype.

Introduction

Acute myelogenous leukemia (AML) is a hematopoietic proliferative disease in which there is clonal proliferation and accumulation of morphologically and functionally immature blast cells, derived from a precursor, transformed hematopoietic marrow cells with the exception of the lymphoid lineage [1, 2]. Acute myelogenous leukemia represents 75–80% of the cases of acute leukemia in adults; in children it occurs in about 15% of all cases [1, 3].

Chromosomal instability manifested in the form of chromosomal aberrations is a classic marker of many malignancies derived from the hematopoietic system [4]. The presence of chromosomal aberrations correlates with morphological features and clinical course of leukemia [5, 6]. In 40–49% of AML patients cryptic structural changes of chromosomes are present, undetectable by means of standard karyotyping, which results in false-negative cytogenetic test results. Patients with such a normal karyotype (cytogenetically normal AML – CN-AML) represent the largest proportion of AML and are classified in the intermediate risk group, together with carriers of t (9;11) and trisomy 8 (+8) [7]. To better characterize the prognosis in this patient group, it is important to study the *FLT3* gene (locus 13q12) and *NPM1* gene (locus 5q35) mutational status.

FLT3

The *FLT3* gene encodes a receptor tyrosine kinase (fms-like tyrosine kinase 3 – FLT3). Tyrosine kinases are enzymes that direct the phosphorylation of proteins involved in cellular signal transduction. FLT3 receptors are present on the surface of early hematopoietic progenitor cells [8].

The FLT3 ligand through its FLT3 receptor plays an important role in the proliferation and differentiation of hematopoietic progenitor cells as well as in the pathogenesis of AML [4, 9]. It is mutated in about 25% of AML patients (all FAB subtypes), either by internal tandem duplications (ITD) of the juxtamembrane domain or by point mutations usually involving the kinase domain (KD) [10, 11]. Both types of mutation constitutively activate FLT3, and autophosphorylation of FLT3 receptor activates the intracellular signal pathways responsible for proliferation [9]. Many studies have shown that AML patients with *FLT3*-ITD mutations have poor cure rates due to relapse [12, 13].

NPM1

The *NPM1* gene contains 12 exons that encode three isoforms of protein – nucleophosmin (NPM), a phosphoprotein with molecular weight of 37 kDa. It belongs to the chaperone protein family involved in, among other actions, the formation of ribosomes and the distribution of centromeres. It plays a key role in post-transcriptional processing of rRNA and ribosomal submission [14, 15]. Nucleophosmin protein maintains genomic stability and controls mechanisms of DNA repair and duplication of centrosomes during mitosis [16]. NPM also interacts with tumor suppressors such as TP53 and ARF [17].

To date, about 50 mutations of the *NPM1* gene have been found in AML [18]. They are the most common cryptic alterations in CN-AML, which are associated with distinct molecular, pathological and clinical characteristics. The majority of *NPM1* mutations in patients with AML have a fixed location in exon 12; they are mostly insertions and deletions [19]. *NPM1*-mutated AML was included in the 2008 World Health Organization (WHO) classification of lympho-hemopoietic neoplasms as a provisional entity.

Identification of cryptic genome alterations allows for more precise stratification of AML patients into risk groups. This is why we decided to check for the aforementioned lesions in patients living in southeastern Poland.

Material and methods

The study group consisted of 50 consecutive patients (19 women and 31 men) aged between 18 and 74 years, hospitalized at the Department of Hematooncology and Bone Marrow Transplantation, Medical University of Lublin in the years 2008–2012. The preliminary diagnosis of AML was based on standard FAB (French-American-British) criteria. The follow-up covered a period of 3 years (36 months).

With informed consent (approval number of the University Ethics Committee: KE-254/24/2011) the bone marrow aspirates were collected to heparin and EDTA collection tubes (Sarstedt, Germany), to be used for cell culture and for DNA isolation, respectively. Twenty-four and 72-hour unstimulated cell cultures were terminated conventionally, then GTG and RHG banded chromosomes were analyzed. Cytogenetic evaluation in selected cases was complemented by the fluorescent in situ hybridization (FISH) technique.

FISH was performed according to the manufacturer's protocol with the probes fort(15;17)(*PML*/*RARα*), *EVI1*, *MYC*, *MLL*, *TP53* and a probe specific for centromeric sequences of chromosome 8 (all from Vysis, Abbott Laboratories, USA).

Determination of the *FLT3* internal tandem gene duplication

For this purpose, PCR was performed on 150 ng of patients' DNA with primers flanking the *FLT3* gene fragment comprising exon 14 and 15 (F: 5'-GCAATTTAGGTATGAAAGCCAGC-3', R: 5'-CTTTCAGCATTTTGACGGCAACC-3', Genomed, Poland) [20]. HD polymerase (2.5 U/ml) from Clontech (Clontech, USA) was used for amplification. The reaction conditions were as follows: i. initial denaturation – 95°C 5 min, ii. 35 cycles of 95°C 30 s, 66°C 30 s, 72°C 30 s, iii. final elongation – 72°C 5 min. The resulting PCR product was electrophoresed in 3.5% agarose gel at 7.5 V/cm for about 60 min.

NPM1 mutation status study

The determination of all possible mutations in exon 12 of the *NPM1* gene was performed using direct sequencing. Primer sequences were selected and the procedure of sequencing was performed according to Döhner *et al.* [21], except for modified thermal conditions of the first PCR reaction aimed at amplifying the template for sequencing reactions: i. initial denaturation – 95°C 5 min, ii. 35 cycles of 95°C 25 s, 57°C 30 s, 72°C 1 min, iii. final elongation – 72°C 5 min. HD Advantage polymerase (Clontech, USA) was used for PCR. Amplification products were sequenced using an ABI PRISM 3130 Genetic Analyzer.

Statistical analysis

Statistical importance was calculated by Fisher's exact test (Statistica v.10.0, StatSoft).

Results

The studies using classical cytogenetics showed chromosomal aberrations in 32 (64%) patients. The most common aberrations included deletion of 7q, 5q, 17p, and trisomy of chromosome 8. In 18 (36%) cases no changes in the karyotype were found by conventional karyotyping (cases defined as CN-AML) (Table I). Implementation of the FISH technique allowed for the confirmation of translocation t(15;17) (*PML/RAR*α) in 2 patients; in one patient this aberration was detected only by FISH. Other alterations identified by FISH included: addition of 3q26.1-q26.3 (*EVI1* gene amplification) in one patient, addition of 8q23-q24.3 (*MYC* gene amplification) in 1 patient, addition of 11q23 (*MLL* gene amplification) in 1 patient, and deletion of 17p13.1 (*TP*53) in 6 (12%) patients (Table I).

Molecular studies of mutations of the *FLT3* gene showed the presence of internal tandem duplication in 4 (8%)patients with AML. In all cases only one allele was affected (Figure 1). *FLT3*-ITD mutation was identified in 1 patient with normal karyotype (CN-AML, FAB M1) and in 3 patients with single lesions in the karyotype (2 classified as M4 and 1 as M1) (Table I).

Studies of mutations in exon 12 of the *NPM1* gene showed the presence of type A mutation (956dupTCTG, the tetranucleotide duplication of TCTG at positions 956 to 959 of the reference sequence – GenBank accession number NM_002520) in three patients with normal karyotype; two of them were classified as M5 and one as M4 according to FAB (Figure 2).

The mutation was present only in patients with normal karyotype, but this difference was not statistically significant (p = 0.0826).

Discussion

FLT3

There are two categories of mutations of the *FLT3* gene. The first group is described as *FLT*-length mutations (*FLT3*-LM), which are heterogeneous, and may present as insertions, deletions, and so-called internal tandem duplications (ITD) [9]. *FLT*-ITD usually occurs within exon 14 or 15, and is usually the result of amplification of the region comprising 3 to 400 base pairs [10]. The second group includes missense point mutations, which lead to an increase in the kinase activity [9]. Nucleotide substitutions typically occur within the intracellular tyrosine kinase domain (*FLT3*-TDK). Both types of mutations (*FLT3*-LM and missense point mutations) cause FLT3 receptor autoactivation.

Multicenter studies have shown a direct correlation between *FLT*-ITD mutations and risk of relapse in patients from the intermediate cytogenetic risk group.

In our studies, *FLT3*-ITD mutation was found in 4 (8%) patients. Studies by numerous authors have shown that this type of mutation is present in 15–35% of patients with AML [14, 22, 23]. The frequency of *FLT3* mutations increases with age, but *FLT3*-ITD in older AML patients (aged 60 and more) has little effect on the outcome, due to the presence of other adverse changes, such as 11q23 rearrangement [1, 11]. In our material, *FLT3*-ITD mutations occurred in patients aged over 50 years. *FLT3*-ITD mutation coexisted with a single lesion in the karyotype in 3 patients, it was also found in one patient with a normal karyotype.

Falini *et al.* [24], while examining a group of 591 AML patients, observed that *FLT3*-ITD mutations occur twice as often in the cases of CN-AML with simultaneous *NPM1* mutations than in the cases without this mutation. However, in our material, no coexisting *FLT3*-ITD and *NPM1* gene mutations were observed.

The results of clinical studies have shown a direct correlation between *FLT3*-ITD mutations and the risk of disease relapse and shorter survival time in AML patients with normal karyotype. In our study, 2 patients (P34 and P35) with internal tandem duplication of *FLT3* died during the 3-year follow-up.

In acute promyelocytic leukemia (M3 according to FAB subtype), a higher incidence of *FLT3* mutations is observed [25]. In our study, *FLT3*-ITD mutations were found only in patients with AML classified as subtype M1 and M4.

Peng *et al.*, examining a group of 150 AML patients, demonstrated a correlation between the presence of *FLT3*-ITD mutation and the increased number of leukocytes in blood, increased bone marrow blast cell count and increased activity of lactate dehydrogenase [26]. In addition, they sug-

Table I. Clinical and molecular characteristics of patients with AML

No.	Age	Gender	FAB subtype	WBC [G/l]	Survival [months]	Karyotype	FLT3-ITD	NPM1
							Mutation	
1	67	F	M4	2.63	1	41-46,XX,der(3)del(3)(p21)t(3;15) (q27;q15),-4,del(5)(q15q35),-15,-17,-18,-21,+mar1,+mar2,+mar3,+1~2mar [cp14]/44~47,sl,+19[cp4]/40~41,sl,del(7) (p11)[cp2] FISH del *TP53*	–	–
2	57	M	M4	12.63	43	47,XY,+8,del(16)(q22)[7]/48,sl, +mar[1]/46,XY,+8,-16[3]/46,XY[10]	–	–
3	29	F	M3	0.80	36	46,XX[14]/46,XX,del(17)(q?)/46,XX,t(14;17)(q?;q?) FISH t(15;17)	–	–
4	48	M	M4	16.10	4	36~42,add(X)(q2?6),-Y,-3,-5,+6,del(7)(q22), add(11)(q2?5),add(12)(p1?1),-13,add(13)(p13),-14,-15,-16,-17,-17,-18,-20,+der(?)t(?;5)(?;q11),+der(?)t(?;13)(?;q12),+4~8mar[cp10]/46,XY[3] FISH amp *MLL;* del *TP53*	–	–
5	50	M	M1	1.50	5	82~94,XXYY,-2,+mar[cp9]/46,XY[4]	–	–
6	57	M	M4	3.90	4	46,XY,i(7)(q10),-13,-15,+mar1,+mar2[5]/46,XY[8]	–	–
7	35	M	M4	11.60	8	43~47,XY,-3,der(4)add(4)(p1?4)add(4) (q3?5),-5,+add(8)(p11orp21),-10,add(11)(q13),-14,-16,-17,der(17), add(17)(p12)add(17)(q2?5),-18,-20,-21, -22, +4~8mar[cp20]	–	–
8	48	F	M5	37.40	11	40~47,XX,del(5)(q13),-8,-17,-18,del(20)(q11.2),-21,+mar1,+mar2, +1~5mar[cp19]	–	–
9	21	F	M4	73.00	10	46,XX,del(9)(q22)[16]/46,XX,-9,+mar[2]	–	–
10	52	M	M6	2.20	30	46,XY	–	–
11	58	F	M2	9.58	25	47~48,XX,+6,+mar,9~34dim[6]/46,XX,4~10dim[5]/46,XX[3] FISH amp *MYC*	–	–
12	56	M	M4	39.80	34	46,XY,add(11)(p15)[11]	+	–
13	52	F	M5	2.41	35	46,XX	–	+
14	53	M	M1	9.60	26	47,XY,+mar [10]/46,XY[10]	+	–
15	51	F	M4	3.22	39	46,XX	–	–
16	72	M	M5	4.50	11	45,XY,der(1)?del(1)(p32),-7[9]/ 46,XY[3]	–	–
17	60	F	M5	4.48	7	45~47,XX,del(15)(q13q33)[5],+8[3],+20[4],-21[7],+mar[cp]/46,XX[7]	–	–

Table I. Cont.

No.	Age	Gender	FAB subtype	WBC [G/l]	Survival [months]	Karyotype	FLT3-ITD	NPM1
							Mutation	
18	55	F	M1	33.00	11	46,XX,-7,+mar[11]/46,XX[6] FISH del *TP53*	–	–
19	64	M	M4	10.38	25	36~44,XY,-5[14],-12[14],-14[6],-15[10],-16[6],-17[10],+22[5],+mar1[14],+mar2[8],+mar3[8], 1~4mar[cp14]/46,XY[2] FISH del *TP53*	–	–
20	60	F	M3	6.20	28	49,XX,+8,+13,t(15;17)(q22;q21),+21[13]/48,sl,-12[4]/46,XX[3] FISH t(15;17)	–	–
21	71	M	M4	5.85	28	46,XY	–	–
22	32	M	M0	26.30	26	42,XY,-3,-7,-17,+mar1[2]/43~45,XY,del(3)(q21),-5,-7,-17,+mar1,+1~2mar[cp4]/42~43,XY,-5,-7,add(8)(q24),-21,-22,+mar1,+1~2mar[cp15] FISH amp *EVI1;* del *TP53*	–	–
23	41	F	M2	25.6	36	44~45,XX,del(4)(q3?1)[13],-10,[13],-12[4],add(16)(q24)[13],+add16(q24) cp[13]/43~44,XX,der(7)t(1;7)(q11;q22)[8],del(4)(q2?1)[8],-10[8],-18[4][cp8]	–	–
24	57	M	M4	12.50	36	46,XY	–	+
25	74	M	M4	1.64	10	47,XY,+21[6]/46,XY[24]	–	–
26	60	M	M4	15.10	33	46,XY	–	–
27	38	M	M4	228.0	26	46,XY	–	+
28	42	M	M2	7.30	25	46,XY,t(8;21)(q22;q22)[20]	–	–
29	63	M	M4	2.76	29	46,XY	–	–
30	48	M	M2	17.86	28	45~46,XY,-20,+mar[7]/46,XY[14]	–	–
31	70	M	M1	263.7	2	46,XY,t(10;12)(q24;p13)[cp10]/46,XY,del(3)(q21),t(10;12)(q24;p13)[5]	–	–
32	46	F	M3	21.60	24	46,XX,t(8;16)(p11;p13)[1]/46,sl,i(8)(q10)[15]/47,sdl1,+mar[4]	–	–
33	46	F	M4	111.8	24	46,XX	–	–
34	52	M	M1	36.50	1	46,XY	+	–

Table I. Cont.

No.	Age	Gender	FAB subtype	WBC [G/l]	Survival [months]	Karyotype	FLT3-ITD	NPM1
							Mutation	
35	65	F	M4	409.5	9	47,XX,+8[3]/46,XX[27]	+	–
36	57	F	M0	2.80	24	51~55,XX,+1,+der(2)t(2;12)(p11;q11),+6,del(7)(p15),+8,+11,-12,+13,add(17)(p12),+19,+1~3mar[cp],46,XX[2] FISH del *TP53*	–	–
37	54	F	M4	2.40	24	46,XX	–	–
38	63	M	M4	3.54	24	46,XY	–	–
39	71	F	M4	58.70	12	45,X,-X[6]/45,XX,-22[5]/46,XX[18]	–	–
40	45	M	M2	2.50	29	46,XY	–	–
41	59	M	M5	4.70	5	46,XY	–	–
42	60	M	M4	46.20	1	46,XY,t(5;7)(q13;p11),?t(X;?)(q13;?)[3]/46,XY[15] FISH t(15;17)	–	–
43	72	M	M4	108.9	11	41~46,X,-Y[3],del(7)(p2?1)[7],add(9)(q2?1)[15],-11[11],add(11)(q1?3)[4], +1~3mar[16][cp20]	–	–
44	40	M	M4	15.00	15	46,XY	–	–
45	36	F	M3	2.24	12	46,XX,t(15;17)(q22;q21)[15]/46,XX[5] FISH t(15;17)	–	–
46	39	M	M4	5.30	25	46,XY,t(9;22;14) FISH BCR/ABL fusion genes (93%)	–	–
47	54	M	M5	213.5	21	46,XY	–	–
48	27	M	M1	21.10	25	46,XY,del(9)(q21q22)[16]/46,XY[6]	–	–
49	31	M	M1	15.10	4	46,XY	–	–
50	30	M	M0	144.2	8	46,XY	–	–

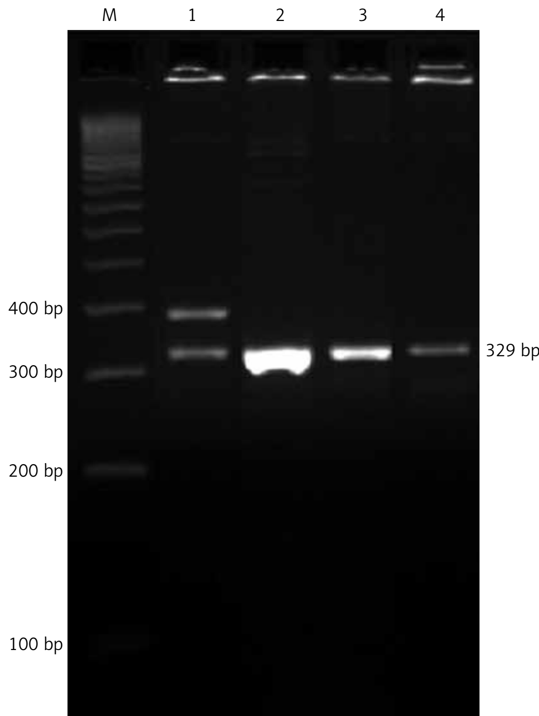

Figure 1. PCR product analysis of *FLT3* internal tandem duplication

M – marker, 1–3 – AML cases, 4 – normal control sample. Note the FLT3-ITD mutation of one allele in patient 1.

gested that *FLT3*-ITD might be an independent prognostic factor in acute myeloid leukemia [26]. In the current study we observed an increased number of white blood cells in three patients with *FLT3*-ITD, which confirms their results.

NPM1

NPM1 mutations substantially affect the course of AML. They include insertions or deletions resulting in partial or complete loss of tryptophan residues. Abnormal protein encoded by *NPM1* changes the location from nuclear to cytoplasmic, which contributes to the development of proliferative disorders [27]. *NPM1* mutations are relatively commonly observed in patients with AML. The majority of *NPM1* mutations occurring in AML involve exon 12; some rare mutations were described in exons 9, 10 and 11 [28, 29]. The most common type of *NPM1* mutation in AML is "type A" mutation, reported in 75–80% of cases [24, 30]. *NPM1* mutations in AML patients are associated with normal karyotype (CN-AML) and are rarely found in patients with chromosomal aberrations [19].

In our research, conducted over 3 years, *NPM1* mutations were observed in 3 (6%) patients. During the follow-up we did not record deaths in patients with *NPM1* mutations. It is known that the presence of *NPM1* mutation augurs favorably and is associated with higher rates of complete remission [29].

In patients with CN-AML and *NPM1* mutations, relapse-free survival is longer in comparison with what we observed in patients with *NPM1* mutations [1].

Backer *et al.* in 2010, studying a group of 148 patients aged ≥ 60 years, found that the prognostic significance of *NPM1* mutations increases with age, especially in older patients. Those patients who have a mutation in *NPM1*, especially without concomitant *FLT3*-ITD mutation, show significantly better prognosis than patients without mutations in the *NPM1* gene [31]. Similar conclusions have been presented by many authors [32, 33].

In our study, we observed the occurrence of mutations in this gene only in patients with normal karyotype, and their average age was 49 years.

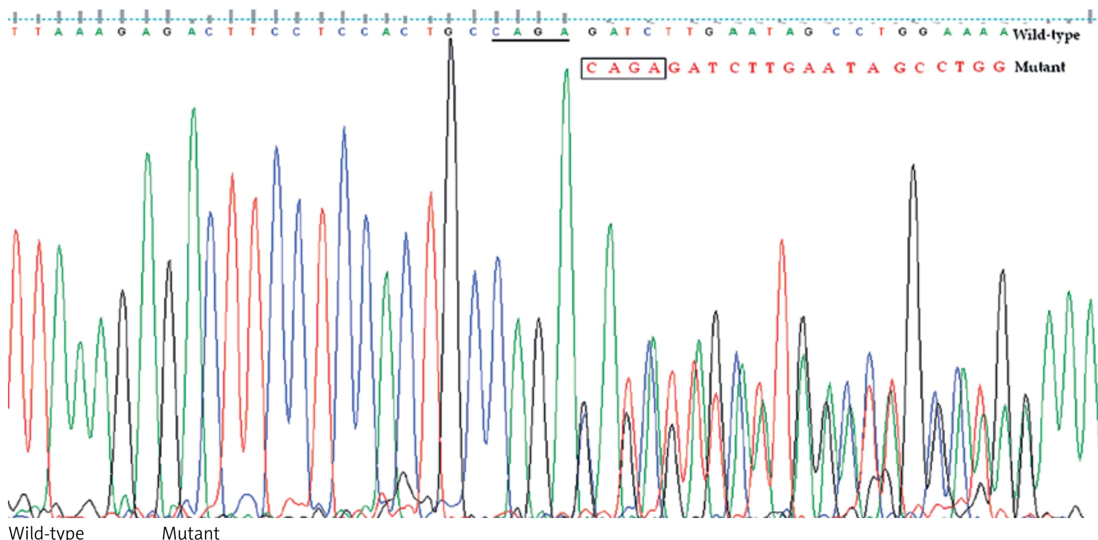

Figure 2. Sequencing analysis of exon 12 of the NPM1 gene. Note type A mutation (reverse direction dupCAGA; forward direction dupTCTG)

NPM1 mutation is usually present throughout the leukemic cell population, demonstrating its essential role in carcinogenesis [24]. It is stable in the course of the disease and its presence is detected also at relapse even a few years after the initial diagnosis [34]. Mutation reversion is extremely rare and sometimes is associated with a change in karyotype [18, 19, 34].

Coexistence of *NPM1* and *FLT3*-ITD mutations is relatively common, found in approximately 18% of patients with CN-AML [35]. Thiede *et al.* [19] found that in patients with *NPM1* and co-existing *FLT3*-ITD, the *NPM1* mutation preceded the occurrence of *FLT3*-ITD.

In our study group, patients carrying both *FLT3*-ITD and exon 12 *NPM1* mutation were not found. The studies by Schneider *et al.* [36] in a group of 1321 patients with CN-AML showed the lower incidence of *FLT3*-ITD and *NPM1* mutation with age in adult patients. Furthermore, lack of mutations in *NPM1* and *FLT3*-ITD in the elderly suggested that other molecular and clinical risk factors might influence the outcome in this age group.

Our findings in a relatively small group of AML patients from southeast Poland confirm other reports of a better prognosis for CN-AML patients with *NPM1* mutation. In the case of our patients with *FLT3*-ITD mutation its association with older age and shorter survival time was also confirmed. As the incidence of both mutations in our study group was lower as compared to other reports, studies on larger cohorts are required to confirm these results.

Acknowledgments

This project was supported by the National Center of Science (NCN) grant NN 402187035 to DK.

This project was supported by the National Center of Science (NCN) grant NN 402187035 to DK and grant MNmb 232 to Szymon Zmorzyński.

Conflict of interest

The authors declare no conflict of interest.

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